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SHORT REPORT

Blockage of heregulin expression inhibits tumorigenicity and metastasis of breast cancer

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The growth factor heregulin (HRG), expressed in about 30% of breast cancer tumors, activates the *erb*B-2 receptor via induction of heterodimeric complexes of *erb*B-2 with *erb*B-3 or *erb*B-4. HRG induces tumorigenicity and metastasis of breast cancer cells. Our investigation into whether HRG is a factor likely to promote tumor formation independently of *erb*B-2 overexpression concludes that blockage of HRG expression suppresses the aggressive phenotype of MDA-MB-231 breast cancer cells by inhibiting cell proliferation, preventing anchorage-independent growth, and suppressing the invasive potential of the cells *in vitro*. More importantly, we observed a marked reduction in tumor formation, tumor size, and a lack of metastasis *in vivo*. These studies were achieved by blocking HRG expression in MDA-MB-231 cells using an HRG antisense cDNA. In the search for the mechanism by which blockage of HRG reverts this aggressive phenotype, we discovered that the cells in which HRG is blocked exhibit a marked decrease in *erb*B activation and a significant reduction in MMP-9 activity, demonstrating a direct causal role in HRG induction of tumorigenicity. Our study is the first report and serves as a proof of the concept that HRG is a key promoter of breast cancer tumorigenicity and metastasis independently of *erb*B-2 overexpression and should be deemed a potential target in developing therapies for breast cancer.
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Growth factors and their receptors have been implicated in playing an important role in the development and progression of cancer. It is known that signaling from the epidermal growth factor receptor (EGFR) family of tyrosine kinase receptors (HER1/EGFR, HER2/*erb*B-2, HER3/*erb*B-3, and HER4/*erb*B-4) is involved in growth regulation of breast cancer cells (Stern, 2000). The role of EGFR in certain types of cancer is well established. Amplification of the *erb*B-2 gene is found in 20–30% of breast cancer patients and correlates with a poor prognosis (Slamon *et al*., 1987), but the clinical relevance of *erb*B-3 and *erb*B-4 has yet to be determined. Unlike other members of the EGFR family, the ligand for *erb*B-2 has not yet been identified. However, *erb*B-2 can be activated by its own overexpression and homodimerization (Pierce *et al*., 1991), or can be transactivated by heregulin (HRG) (Lupu *et al*., 1992a, b). The HRG/*neu* differentiating factor (NDF) family of polypeptide growth factors binds either to *erb*B-3 or *erb*B-4 receptors and indirectly induces activation of *erb*B-2 through the formation of *erb*B-2: *erb*B-3 or *erb*B-2: *erb*B-4 heterodimers (Lupu *et al*., 1990; Plowman *et al*., 1993; Sliwkowski *et al*., 1994). It is believed that the effects of HRG are mediated primarily through *erb*B-2, because functional blocking of *erb*B-2 inhibits HRG-induced cellular proliferation and transformation (Alimandi *et al*., 1995). Numerous studies from others and from our laboratory have shown that biological response to HRG seems to depend directly upon the level of *erb*B-2 expression in breast cancer cells. In cells that overexpress *erb*B-2, low concentrations of HRG exhibit mitogenic stimulation, whereas high levels of HRG or constitutive expression of HRG induce growth arrest, cellular differentiation, or apoptosis (Lupu *et al*., 1992a; Bacus *et al*., 1992; Guerra-Vladusic *et al*., 1999; Tripathy and Benz, 1992). In contrast, HRG at all concentrations stimulates proliferation in breast cancer cells that express low levels of the *erb*B-2 receptor (Lupu *et al*., 1995).

Although overexpression of *erb*B-2 is a marker of poor prognosis in breast cancer, 70% of breast cancers overexpressing *erb*B-2 are characterized as noninvasive intraductal carcinoma (Paik *et al*., 1990). This indicates that *erb*B-2 alone may not be sufficient for developing metastatic phenotypes and may require additional regulators for tumor progression. Interestingly, our data have shown that HRG is overexpressed in nearly 30% of breast cancer tumor biopsies that do not overexpress *erb*B-2 (Cardillo *et al*., 1995). We have further shown that HRG expression in breast cancer cell

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lines and in breast tumor biopsies is inversely correlated with overexpression of *erb*B-2 and expression of estrogen receptor (ER) (Cardillo *et al*., 1995). Our previous data demonstrate that HRG promotes tumorigenicity and metastasis of breast cancer cells that do not overexpress any of the *erb*B receptors (Tang *et al*., 1996).

Significantly, the population of breast tumors that overexpresses HRG is distinct from the population overexpressing *erb*B-2, (Cardillo *et al*., 1995). One such *in vitro* system manifesting this clinical observation is the ER-negative MDA-MB-231 human breast cancer cell line. These cells overexpress HRG, and are highly invasive *in vitro*, and tumorigenic and metastatic *in vivo*. This cell line expresses low levels of *erb*B-2 and *erb*B-3, (Cardillo *et al*., 1995). We have further shown that antibodies generated against HRG markedly reduce *in vitro* growth, motility, and invasion of breast cancer cells that overexpress HRG, indicating that HRG is essential for breast cancer cell proliferation, motility, and invasion *in vitro* (Hijazi *et al*., 2000). In the light of these observations, we hypothesize that HRG is a tumor-promoting factor that acts independently of *erb*B-2 overexpression. In this scenario, blockage of HRG expression will result in cell growth inhibition, reduction of tumorigenicity, and suppression of metastasis. We hypothesize that these events will occur in cells (or tumors) that express low levels of *erb*B-2 and high levels of HRG, such as the MDA-MB-231 breast cancer cells. It is important to look at other extremely aggressive tumors that do not overexpress *erb*B-2 and that require other biological therapies. Results from our present study strongly provide the proof of the concept that it should be plausible to target HRG for a large population of breast cancer patients (about 30%), those whose tumors overexpress HRG and express low levels of *erb*B-2.

To block HRG expression, a eukaryotic expression vector (pRC/CMV) was constructed with the HRG-β2 cDNA (amino acids 1–426) oriented from 3′ to 5′ end, that is, in an antisense direction, and subsequently transfected into MDA-MB-231 cells. Several HRG antisense (HRG/AS) clones were isolated and the presence of antisense HRG mRNA was confirmed by the RNAse protection assay (data not shown). Also generated were multiple clones of vector-transfected MDA-MB-231 (231/V) cells, all of which behaved similarly to the wild-type cells. Two MDA-MB-231 HRG/AS clones (C6 and C31) and one vector clone (231/V) are characterized further. The HRG protein expression was determined by Western blot analysis using an anti-HRG rabbit polyclonal antibody generated in our laboratory (Tang *et al*., 1996). Conditioned media from C6, C31, and 231/V were collected and the HRG protein was purified by heparin chromatography as previously described (Lupu *et al*., 1992b). Expression of the 45 kDa HRG protein was significantly reduced by 25–30-fold in the C6 cells and was low to undetectable in the C31 cells, as compared to the 231/V cells (Figure 1a). The biological activity of the remaining HRG expressed in clones C6 and C31 was next examined (as previously described – Guerra-Vladusic *et al*., 1999) by HRG's ability to induce p185 tyrosine phosphorylation in MDA-MB-453 cells, which overexpress *erb*B-2 and express low levels of *erb*B-3 and *erb*B-4. Our results demonstrate that the ability of the remaining HRG in the HRG/AS cells to induce p185 tyrosine phosphorylation was extremely low to nearly undetectable, comparable to that in untreated cells (Figure 1b). In contrast, the 231/V cells induced *erb*B receptor tyrosine phosphorylation to an extent similar to cells treated with exogenous HRGβ1 (Figure 1b). As expected, the decreased level of HRG expression in the HRG/AS clones correlates with the inability of their conditioned media to induce *erb*B activation in MDA-MB-453 cells. These results demonstrate that the expression of HRG/AS DNA specifically and effectively blocks translation of the HRG mRNA into protein, and therefore

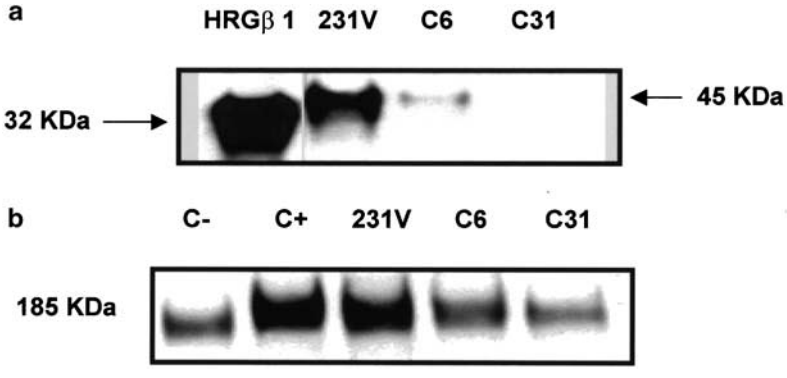

Figure 1 HRG expression and its ability to induce *erb*B-2 tyrosine phosphorylation are diminished in the HRG-AS clones. (**a**) Partially purified HRG from the conditioned media (CM) collected from vector-transfected cells (231/V) and HRG/AS-transfected cells (C6 and C31) was detected by Western blot analysis with an antibody against HRG that recognizes a 45-kDa protein (Tang *et al*., 1996; Guerra-Vladusic *et al*., 1999). Recombinant HRG-β1 protein (32 kDa) was used as a positive control. (**b**) Induction of p185 tyrosine phosphorylation of the *erb*B receptors in MDA-MB-453 cells. Partially purified HRG derived from the CM collected from 231/V, C6, and C31 cells (**a**) was used to determine p185 tyrosine phosphorylation by Western blot analysis using an anti-phosphotyrosine antibody (Tang *et al*., 1996). MDA-MB-453 cells were treated in the presence or absence of HRG-βl protein, denoted as positive (C+) and negative (C−) controls, respectively

significantly reduces HRG expression and its biological activity.

MDA-MB-231 is one of the most aggressive breast cancer cell lines, and these cells grow rapidly *in vitro*. Thus, the effect of blocking HRG expression on the anchorage-dependent growth of the cells was examined first. As shown in Figure 2a, the proliferation rate of HRG/AS clones C6 and C31 was decreased 35–50%, as compared to the 231/V cells. It is also known that HRG-overexpressing cells, such as MDA-MB-231, grow in an anchorage-independent manner. To determine whether inhibition of HRG expression had any effect on the ability of cells to grow as anchorage-independent cells, the HRG/AS and 231/V cells were tested in the soft agar assay as previously described (Tang *et al*., 1996). Colonies with size between 60 and 100 μm were quantified using a soft agar colony counter. As shown in Figure 2b, although the total number of colonies of the C6 cells was not significantly different from that of the 231/V cells, the C6 colonies were generally at the smaller end of the size range (about 60 μm). On the other hand, the C31 cells showed a 50% reduction in anchorage-independent growth, as compared to the 231/V cells. Therefore, the decrease in HRG expression in MDA-MB-231 cells not only inhibits the anchorage-dependent growth, but also decreases the ability of the cells to grow in anchorage-independent manner.

MDA-MB-231 cells display stellar-like growth patterns in the Matrigel outgrowth assay (Sommers *et al*., 1994). We have previously shown that an HRG-neutralizing antibody prevents MDA-MB-231 cells from developing stellar-like patterns in these assays (Hijazi *et al*., 2000). Thus, we predicted that the HRG/AS clones would no longer reveal stellar-like patterns as seen in MDA-MB-231 cells. In this study, cells were plated on a Matrigel layer and grown for 7 days as previously described (Hijazi *et al*., 2000). Neither of the HRG/AS clones was able to grow with stellar-like patterns as compared with the 231/V cells (Figure 2c). The C6 cells formed small foci, and migrated through the surrounding matrix. The C31 cells were not able to form proliferative foci, nor did they migrate through the Matrigel. In contrast, the 231/V cells formed large foci with stellar-like patterns, moving outward across the Matrigel as they replicated, and forming wide pathways of multiple cells through the surrounding matrix extensively (Figure 2c). These results imply that HRG is necessary for breast cancer growth and invasiveness. We then tested the ability of the HRG/AS cells to migrate and invade using the Boyden chamber assay as previously described (Hijazi *et al*., 2000; Tsai *et al*., 2000) Chemomigration is tested using a collagen matrix, and chemoinvasion is assessed with a Matrigel matrix. Both chemomigration and chemoinvasion of the C6 cells was reduced by 50% as compared with the 231/V cells; in the C31 cells, chemoinvasion was reduced by 75% and chemomigration was completely abolished, when compared with the vector cells (Figure 2d). As expected, the 231/V cells migrated and invaded rapidly through both the collagen and Matrigel matrices (Figure 2d).

It is noteworthy that HRG protein expression is clearly decreased in clone C6, but not completely blocked as in clone C31 (Figure 1a). The threshold expression of HRG in clone C6 appears sufficient to induce *erb*B-activation (Figure 1b). Moreover, the effect of HRG threshold expression is more evident for observed intermediate levels of anchorage-independent growth in clone C6 (Figure 2b), in which a marked decrease in colony size was more evident than a decrease in colony number, as compared with 231 V cells. Furthermore, the C6 cells displayed moderate reduction in their outgrowth ability in Matrigel, in chemomigration, and in emoinvasion, as compared with the 231 V and C31 cells (Figure 2c, d). All of these data indicate that a steady-state decrease in the aggressiveness of MDA-MB-231 cells depends directly upon the threshold level of HRG. The data presented to this point, clearly demonstrate that blockage of HRG expression reduces cellular proliferation, inhibits anchorage-independence, blocks Matrigel outgrowth, and decreases chemoinvasive and chemomigration behavior. All together, these data demonstrate that HRG is necessary to induce aggressive phenotypes of the MDA-MB-231 cells.

To assess the effect that blockage of HRG has *in vivo*, the HRG/AS cells were inoculated into the mammary fat pads of 3–4-week-old athymic nude mice. At 4 weeks after cell inoculation, we observed that the C6 and C31 cells showed a significant decrease in tumor size and in total weight, as compared with the 231/V cells, which develop largely vascularized tumors similar to the human invasive breast carcinomas in athymic nude mice (Figure 3). The variability in tumor size among each group was not significant. However, the HRG/AS-derived tumors were extremely small (Figure 3) and did not appear vascularized (data not shown). The mice containing the 231/V-derived tumors were killed 4 weeks after cell inoculation, since the tumors reached the largest size allowable. Mice that contained the HRG/AS-derived tumors were kept for an additional 8 weeks, during which time they did not show a significant change in tumor intake and tumor size. It is known that MDA-MB-231 cells are not only tumorigenic but also metastatic. As expected, the 231/V cells that developed into large tumors were readily metastatic, and the metastatic foci appeared in the liver and the lung (Figure 3b). In contrast, neither the C6- nor the C31-derived tumors metastasized even 12 weeks after the initial cell inoculation. Our *in vivo* results are in agreement with the *in vitro* data. The data presented here without a doubt demonstrates that HRG controls the tumorigenic and metastatic potential of many breast cancer cells in vivo. It appears that blockage of HRG blocks the signaling machinery necessary for these cells to grow, invade and metastasize.

Our studies lead us to conclude that HRG is a tumor-promoting factor, expression of which is critical for the progression of breast carcinomas. The data presented here support the notion that the behavior of breast cancer cells in culture correlates to varying levels of HRG expression (Hijazi *et al*., 2000). It appears that the different threshold levels of HRG expression in clones

C6 and C31 promote slightly different *in vitro* phenotypes (Figures 1 and 2). Although there is a minor difference in the threshold HRG expression in each HRG/AS clone, the *in vivo* behavior of all the HRG/AS cells is similar (Figure 3). Our results are of great importance for the development of therapies that will be

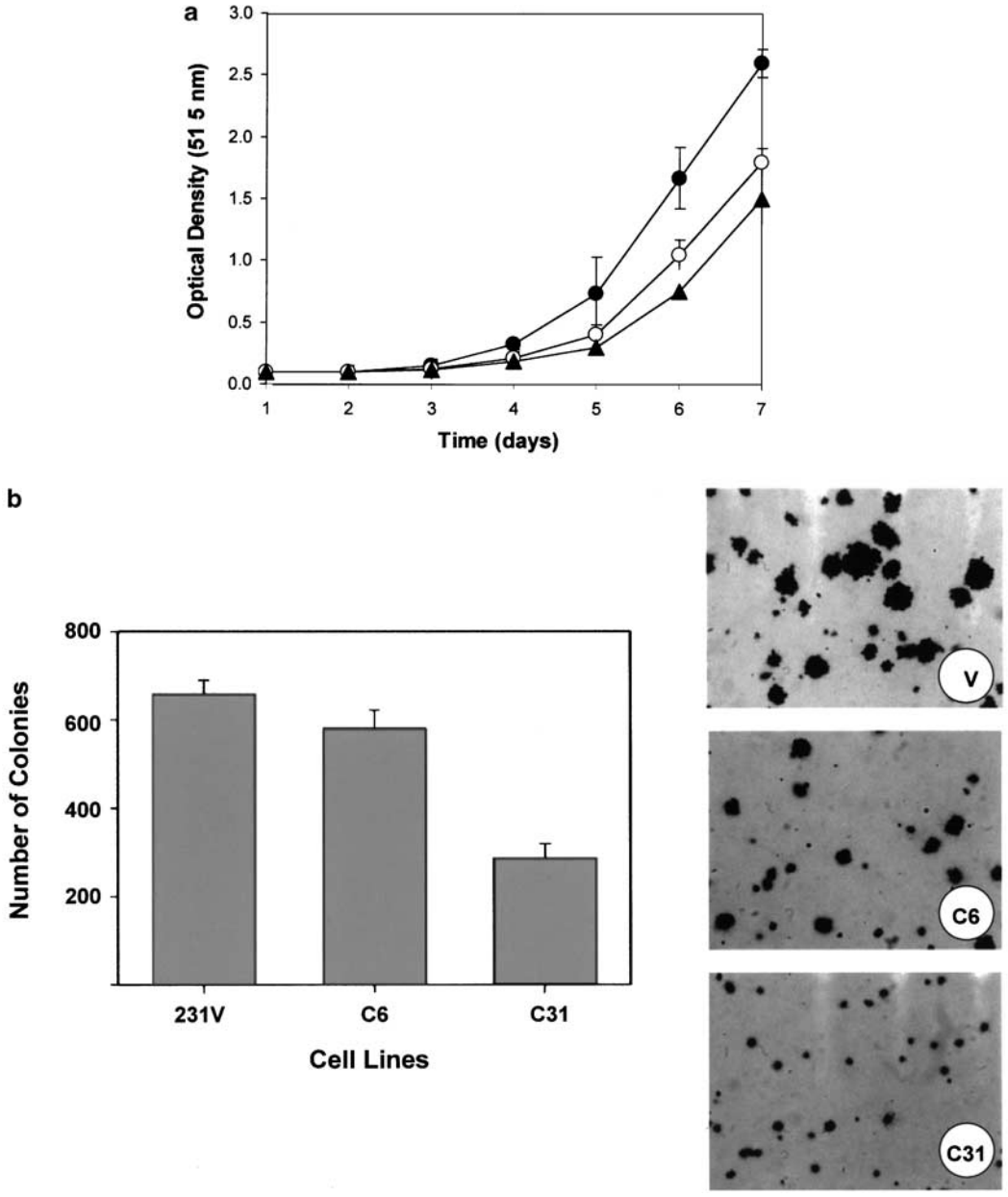

Figure 2 Obstruction of HRG expression results in a significant decline in the aggressiveness of MDA-MB-231 *in vitro*. (**a**) Anchorage-dependent growth was decreased in HRG/AS cells. Cells (1000/well), 231/V (•), C6 (○), and C31 (▲), were seeded in 96-well plates. Growth was evaluated over a period of 7 days by fixing cells with trichloroacetic acid, staining with sulforhodamine B, and measuring optical density at 515 nm. (**b**) HRG/AS cells were significantly less clonogenic than the 231/V cells in soft agar. Cells (5000/well) were plated in triplicate in agar layer and grew for 2 weeks at 37°C. Colonies of 60–100 μm were stained with *p*-iodonitrotetrazolium purple and quantified with an AccuCount 2000 automatic colony counter (Grunt *et al*., 1995). (**c**) The pattern of Matrigel outgrowth of the HRG/AS clones was markedly changed as compared with the 231/V cells, from a stellar-like pattern with invasive components in the 231/V to small foci with limited invasive components, if any, in the HRG/AS cells. Cells (25 000/well) were plated in triplicate in Matrigel in a 12-well plate, and microphotographs were taken at day 7 as previously described (Hijazi *et al*., 2000). (**d**) The HRG/AS showed decreased chemoinvasion and chemomigration activities. Boyden chamber assay of HRG/AS clones was performed as previously described (Tsai, *et al*., 2000; Hijazi *et al*., 2000). In brief, cells (20 000 cells/well) were plated in quadruplicate in the upper chamber of a 48-well Boyden chamber onto polycarbonate filters coated with either collagen IV or Matrigel in serum-free media. CM from NIH3T3 fibroblast was used as a chemoattractant in the lower chambers. After incubation for 6 h at 37°C, cells on the top surface were removed, and filters were then fixed, and stained with crystal violet. The number of cells that migrated through the pores was assessed by microscopy. Similar results were obtained from at least four to five independent experiments

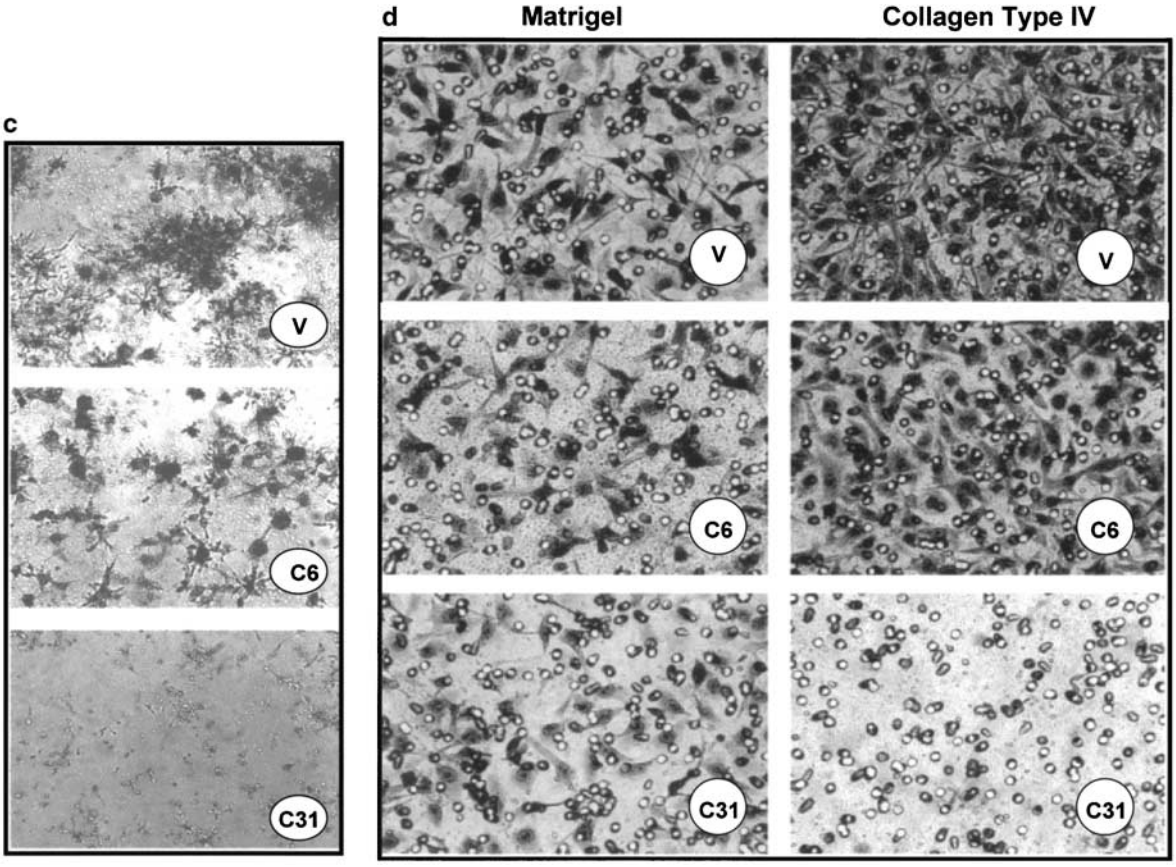

Figure 2 *Continued*

aimed at targeting HRG for use in those breast cancer patients in whom increased expression of HRG is observed. We do postulate that those tumors are likely to develop tumor metastasis. Our initial studies demonstrate that 30% of invasive breast carcinomas express high levels of HRG and its expression is correlated with aggressiveness of the tumor, lack of ER and low expression of erbB-2 (data not shown).

To understand the mechanism by which blockage of HRG expression reverts the aggressive phenotype of MDA-MB-231 cells, we examined whether HRG-mediated signaling pathways are altered in the HRG/AS cells. Expression of the *erb*B-2 and *erb*B-3 receptors was assessed by performing immunoprecipitation under nonreducing conditions, followed by immunoblotting for *erb*B-2 and/or *erb*B-3 receptors using specific receptor antibodies. To evaluate the level of *erb*B-2 and *erb*B-3 tyrosine phosphorylation, immunoprecipitations as described above were followed by immunoblotting for phosphotyrosine using an antiphosphotyrosine antibody. We demonstrate that the level of the *erb*B-2 protein, although low, was unchanged in the 231/V and HRG/AS cells (Figure 4a, top panel). In contrast, the level of *erb*B-2 autophosphorylation was decreased in the C6 and C31 cells, as compared to the 231/V cells (Figure 4a, middle panel). The basal level of *erb*B-3 protein was low to undetectable (data not shown) in all of the cells, and the level of *erb*B-3 tyrosine phosphorylation was markedly decreased in the C6 and C31 cells in comparison with the 231/V cells (Figure 4a, bottom panel). Our data clearly suggest that by blocking HRG expression, a cascade of events leads to a decrease in both *erb*B-2 and *erb*B-3 tyrosine phosphorylation. Tyrosine phosphorylation of *erb*B receptors is one of the signaling events that modulate cell proliferation, tumor formation, and metastatic behavior of breast cancer cells. This is an important observation supported by the previous data that the primary *erb*B heterodimer in human breast carcinomas is *erb*B-2: *erb*B-3, which is correlated with an aggressive phenotype (Chen *et al*., 1996). MDA-MB-231 cells do not overexpress *erb*B-2 and *erb*B-3. From our data, it is clear that the growth and the receptor signaling events in these cells are not dependent upon *erb*B-2 overexpression, but rather on ligand (HRG) induced receptor heterodimerization. Thus, obstruction of HRG expression in MDA-MB-231 cells disrupts HRG-induced heterodimerization between erbB-2 and erbB-3, leading the lower erbB-2 activation. Interestingly, MDA-MB-231 cells express high levels of EGFR (*erb*B-1) and secrete TGFO and EGF, both of which directly activate and phosphorylate EGFR in an autocrine manner. Aggressiveness of

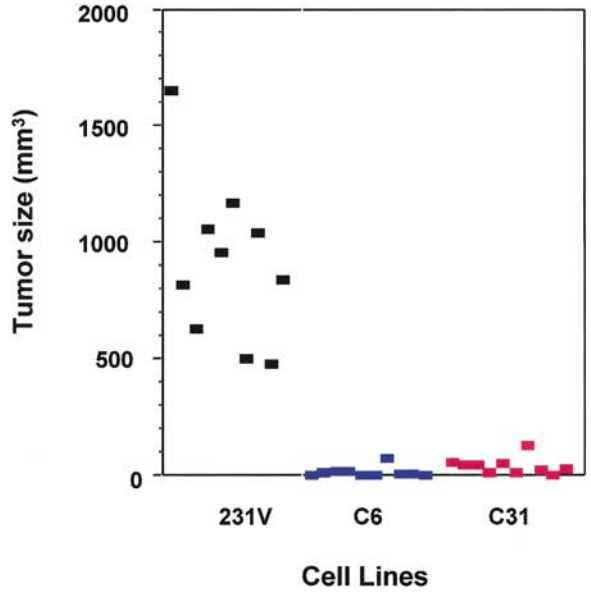

Figure 3 Blockage of HRG expression suppresses tumorigenicity and metastasis of MDA-MB-231 cells *in vivo*. (**a**) Athymic nude mice (3–4-week old) were implanted with 231/V, C6 and C31 cells (5×10^5/site) into the mammary fat pads as described previously (Tang *et al*., 1996). Tumor size was calculated by three-dimensional measurements. The tumors produced by the 231/V cells ranged from 500 to 1600 mm^3, and no significant tumor formation was seen in mice inoculated with the HRG/AS cells (C6 and C31). Studies were performed for 12 weeks; however, all the measurements were performed 4 weeks after inoculation. No significant changes in tumor development were observed in the HRG/AS-inoculated mice. Control 231/V mice were sacrificed after 4 weeks because of the large appearance of the tumors. (**b**) Metastases derived from the primary tumors were observed by H&E staining in the sections of liver (a) and lungs (b) from mice inoculated with the 231/V cells. No metastases were observed in the HRG/AS-inoculated mice. Arrows indicate the detection of human breast cancer epithelial cells

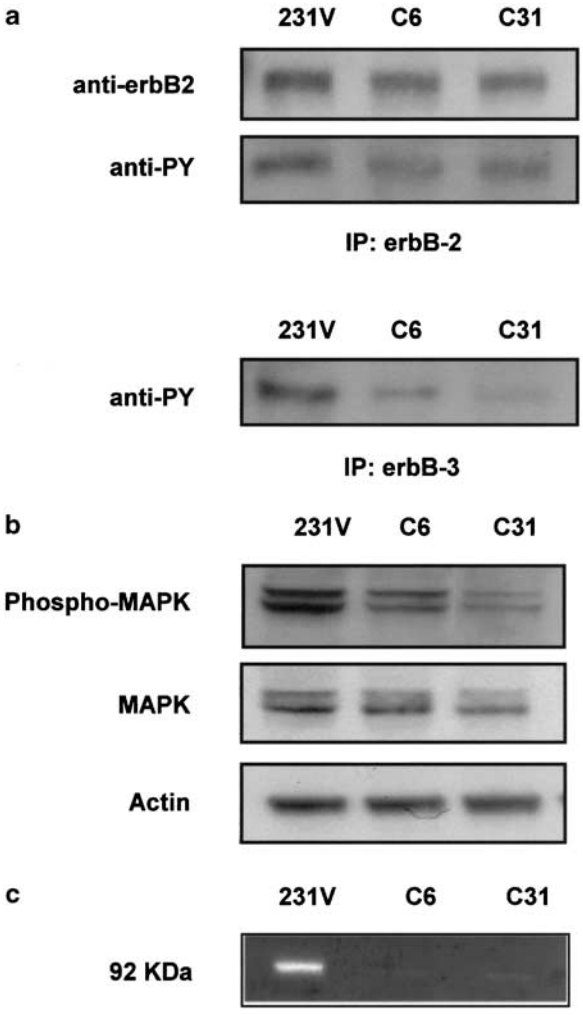

Figure 4 Inhibition of HRG expression causes decreased activation of *erb*B-2, MAPK, and MMP-9 activity in MDA-MB-231 ceils. (**a**) Expression and activation of *erb*B receptors were downregulated in HRG/AS cells. The *erb*B receptors were immunoprecipitated from lysates prepared from 231/V, C6, and C31 cells, and immunoblotted for *erb*B-2 (top panel), *erb*B-2 tyrosine phosphorylation (middle panel), and *erb*B-3 tyrosine phosphorylation (bottom panel). (**b**) Activation of MAPK was decreased in HRG/AS clones. Cells (231/V, C6, and C31) were serum-starved for 24 h. Cell lysates were collected, separated on SDS–PAGE, and immunoblotted with antibodies against phosphorylated MAPK (top panel), MAPK (middle panel), and actin (bottom panel). (**c**) MMP-9 enzymatic activity was diminished in HRG/AS cells. Conditioned media were collected and concentrated 100× from the 231/V, C6, and C31 cells after 72-h serum starvation. Equal amounts of protein were loaded onto an SDS–gelatin–PAGE and analysed for MMP-9 activity using reverse zymography as described previously (Hua and Muschel, 1996)

MDA-MB-231 cells, however, is not dependent upon EGFR activation. It has been shown that blockage of EGFR/EGF disruption does not lead to reduction of tumor formation in mDA-MB-231 cells, although these cells overexpress EGFR. Namely, EGFR does appear to be a relevant pathway for tumorigenic phenotype of these cells. Furthermore, inhibition of HRG has no effect on EGFR activation (data not shown). This is an extremely important finding, because it is the first indication that blockage of HRG expression inhibits tumorigenicity and abolishes the metastatic processes of the cells by perhaps inhibiting a large cascade of events driven by the erbB-signaling pathway.

Next, we examined MAPK, a downstream effector molecule of the *erb*B receptor tyrosine phosphorylation. It has been shown that activation of *erb*B-2 by HRG leads to breast cancer proliferation, presumably by inducing activation of the MAPK and PI3 K (phosphotidylinositol-3 kinase)/Akt pathways (Sepp-Lorenzino *et al*., 1996; Reese and Slamon, 1997; Lim *et al*., 2000). We then postulated that inhibition of HRG expression would have an impact on the erbB-downstream signaling molecules *Ras*/*MAPK* which may be in part, controlling cell growth and proliferation. We found that phosphorylated MAPK (ppMAPK) was greatly decreased in the C6 and C31 cells by a more than 80% reduction, as compared with the 231/V cells cultured under the same conditions (Figure 4b, top panel). To demonstrate that a decrease in ppMAPK was specific, and not because of a decrease in the total MAPK protein, the levels of the MAPK protein were also assessed. The total MAPK protein levels in the C6 and

C31 cells were not altered as compared to 231/V cells (Figure 4b, middle panel). Protein loading control was shown by using an anti-actin antibody to ensure that equal amounts of protein were analysed (Figure 4b, bottom panel).

Through this, we demonstrate that HRG-induced tumorigenicity and invasiveness is, at least in part, regulated through *erb*B receptor-mediated *Ras*-dependent MAPK pathways. These events have previously been shown *in vitro* and in other model systems, which have demonstrated that HRG promotes cellular proliferation through the *Ras*-dependent MAPK (Sepp-Lorenzino *et al*., 1996). It has also been shown that upon activation by HRG, *erb*B-2 becomes phosphorylated and bound to the SH2 domain of the Grb2 (Lim *et al*., 2000), which in turn leads to activation of MEK and MAPK (Reese and Slamon, 1997). It should be noted that MDA-MB-231 cells express activated Ras. However, it is most likely that nonactivated Ras exists as *a* predominant form (as in most cells), which may be activated through the HRG/*erb*B signaling pathway. Blockage of HRG expression promotes a decrease in *erb*B-3 and *erb*B-2 activation as well as a decrease in activation of the downstream signaling molecules. This is the first report demonstrating that, in fact, HRG is *a* key regulator of these events in breast carcinomas, and that blockage of HRG expression leads to a phenotypic regression, from a very aggressive and metastatic phenotype to a nonaggressive and nonmetastatic phenotype. These changes are solely mediated by the blockage of HRG expression and the impact that this blockage has on erbB-activation.

Matrix metalloproteases (MMPs) have been associated with tumor cell invasion and metastasis (Curran and Murray, 1999). Since we had initially seen (Atlas *et al*., 2003), that HRG induced MMP-9 activity, we postulated that one of the mechanisms by which blockage of HRG caused inhibition of metastasis was perhaps by blockage of an extracellular matrix degrading enzyme, such as the MMP-9. We have also shown that a specific MMP-9 inhibitor blocks the invasive phenotype of HRG-expressing cells, but not cells that do not express HRG (Liu *et al*., 1999; Atlas *et al*., 2003). Thus, we investigated MMP-9 expression and activity in the antisense expressing cells. We demonstrated the MMP-9 expression was not changed between the control and the antisense HRG cells, but a tremendous decrease in MMP-9 activitiy. Assessment of MMP activity was performed using a reverse zymography as previously described (Hua and Muschel, 1996; Lee *et al*., 1996). A striking difference in the MMP-9 activity was found. MMP-9 was low or undetectable in the C6 and C31 cells, as compared with 231/V cells, which secreted high levels of MMP-9 enzymatic activity (Figure 4c). MMP-9 is a metalloprotease that plays a role in the degradation of type IV collagen (gelatin), and has been found highly expressed in breast carcinomas (Himelstein *et al*., 1997). Our results strongly suggest that the enzymatic activity of MMP-9 is associated with HRG expression, and that both are involved in the invasive and metastatic phenotype of MDA-MB-231 cells. Moreover, our results are consistent with previous observations, in which increased production of pro-MMP-9 and secretion of MMP-9 are associated with metastasis induced by activated *Ras*-transformed breast cancer cells (Tsang and Crowe, 2001). MMP-9 is required for this process, because a ribozyme directed against MMP-9 abolishes the ability of the cells to metastasize (Hua and Muschel, 1996). Moreover, it has been shown recently that MMP-9 expression is activated by HRG (Yao *et al*., 2001), and that the *erb*B-2-mediated *Ras*-dependent MAPK pathway is involved in the upregulation of MMPs (Tsang and Crowe, 2001). We have previously demonstrated that HRG promotes tumorigenicity in part via upregulation of an angiogenic factor Cyr61 (Tsai *et al*., 2000), and also via receptor tyrosine phosphorylation and MAPK activation (Atlas *et al*., 2003; Tsai *et al*., 2002). However, this is the first report to demonstrate the importance of MMP-9 activity driven by HRG expression, as well as the function of MMP-9 in promoting metastasis (Atlas *et al*., 2003).

In summary, our present study demonstrates indisputably the crucial role that HRG plays in acquiring an aggressive phenotype of the human breast cancer cells. By effective blockage of HRG expression using stable transfection with an antisense RNA expression vector for HRG, we show nearly complete reversion of the tumorigenic and metastatic phenotype of the MDA-MB-231 cells. These results are of critical significance, endorsing the view that the aggressive phenotypes are not dependent on *erb*B-2 overexpression, but on expression of HRG and erbB receptor activation in several breast cancer cells, as manifested clinically in certain populations of human breast carcinomas. Examples include MDA-MB-231 presented here, and two other breast cancer cell lines HS578 T and BT549, in which the data were very similar (data not shown). Moreover, our results provide evidence into the possible mechanism of blocking HRG action in halting breast cancer progression through inactivation of HRG-induced *erb*B signaling and the subsequent decrease in activated MAPK, MMP-9 activity and possibly AKT activation.

Even though the two distinct breast cancer populations – one overexpressing *erb*B-2 with low levels of HRG, and the other expressing HRG but not *erb*B-2 – may appear to employ the same signaling pathways in growth control, effective treatments of breast carcinomas should be specifically targeted. This is the first report addressing the possibility of targeting HRG in breast carcinomas that do not overexpress *erb*B-2, and demonstrating the critical role of such intervention against HRG. We show clearly here that blockage of HRG expression results in nearly complete inhibition of tumor formation and obstruction of metastasis *in vivo*. Our unique and novel findings furnish a proof of concept and provide new insights into the development of potential therapies targeted at blocking HRG expression and/or its action and thereby halting the progression of breast cancer. This will benefit a large breast cancer population whose tumors overexpress HRG, the 30% of patients who have invasive breast

carcinomas and for whom conventional therapies are known to fail.

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